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Article

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Constitutive serotonin transporter reduction resembles maternal separation with regard to stress-related gene expression

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Author Contributions

JDAO, ISP: study design; JDAO: animal experiment; DS, HDM: RNA isolation; DS, HDM, MV: gene expression analyses; EC: statistical analyses and manuscript draft; JO, IN, ISP: critical revision of the manuscript; all authors: finalization and approval of the content of the manuscript.

ABSTRACT

Interactive effects between allelic variants of the serotonin transporter (*5-HTT*) promoter-linked polymorphic region (5-HTTLPR) and stressors on depression symptoms have been documented, as well as questioned, by meta-analyses. Translational models of constitutive *5-htt* reduction and experimentally controlled stressors often led to inconsistent behavioral and molecular findings, and often did not include females.

The present study sought to investigate the effect of *5-htt* genotype, maternal separation, and sex on the expression of stress-related candidate genes in the rat hippocampus and frontal cortex.

The mRNA expression levels of *Avp*, *Pomc*, *Crh*, *Crhbp*, *Crhr1*, *Bdnf*, *Ntrk2*, *Maoa*, *Maob*, and *Comt* were assessed in the hippocampus and frontal cortex of *5-htt*^{+/-} and *5-htt*^{+/+} male and female adult rats exposed, or not, to daily maternal separation for 180 minutes during the first two postnatal weeks.

Gene- and brain region-dependent, but sex-independent, interactions between *5-htt* genotype and maternal separation were found. Gene expression levels were higher in *5-htt*^{+/-} rats not exposed to maternal separation compared to the other experimental groups. Maternal separation and *5-htt*^{+/-} genotype did not yield additive effects on gene expression. Correlative relationships, mainly positive, were observed within, but not across, brain regions in all groups, except in non-maternally separated *5-htt*^{+/+} rats.

Gene expression patterns in the hippocampus and frontal cortex of rats exposed to maternal separation resembled the ones observed in rats with reduced *5-htt* expression, regardless of sex. These results suggest that floor effects of *5-htt* reduction and maternal separation might explain inconsistent findings in humans and rodents.

Keywords: frontal cortex; hippocampus; maternal separation; serotonin transporter; gene expression

Introduction

Fifteen years have passed since Caspi and colleagues provided evidence of an interaction effect of the serotonin transporter linked polymorphic region (5-HTTLPR) with childhood maltreatment on depressive symptoms¹. An almost perfect fan-shaped relationship was presented. In the absence of maltreatment, all three genotypes (i.e. SS, SL and LL) were associated with a similar probability of depressive episodes, whereas in the presence of maltreatment, the probability was dose-dependently increased by the number of S alleles¹. This has been a milestone finding in psychiatric genetics and has prompted not only several replication attempts, as highlighted by reviews and meta-analyses^{2,3}, but also research on the neurobiological mechanisms behind this interaction⁴⁻⁷.

Twin studies have indeed demonstrated a moderate degree of heritability for depression⁸, thus pointing to the importance of studying not only constitutive but also psychosocial factors and their interactive effects. Of particular importance, especially considering the developmental role of serotonin, are those interactions taking place during sensitive periods of an individual's life, such as during foetal and early life^{9,10}. As the brain does not reach full maturity until young adulthood, its development is sensitive to factors, such as stress, that can program developmental pathways through epigenetic mechanisms^{9,11}. However, we know that not every individual exposed to stress will develop a psychiatric disorder. The genetic make-up, including sex, can set *a priori* susceptibility as well as moderate environmental influences on mental health⁴. Regarding sex, compared to men, women are at higher risk for development of depression or receiving treatment for depression¹². Selective serotonin reuptake inhibitors (SSRI) is the most common pharmacological treatment modality for depression. SSRIs block the reuptake of serotonin from the synaptic space, thereby leading to increased extracellular levels of serotonin¹³. Altogether, the 5-HTTLPR has been suggested to influence not only predisposition to depression⁴ but also response to SSRI therapy¹⁴.

The 5-HTTLPR has been proven to be a functional polymorphism *in vitro*¹⁵; the S allele has been associated with lower promoter activity and mRNA levels, which ultimately can lead to lower serotonin reuptake^{15,16}. To further understanding of the effects of this genotype on the brain, genetically engineered mutations have been created in rodents because they do not have an orthologue of the human polymorphism^{4,17,18}. However, it is important to note that the knock-out homozygous rodents (5-*htt*^{-/-}) embody a condition that does not exist in humans, thus limiting their translational validity. Conversely, heterozygous rodents (5-*htt*^{+/-}) still display serotonin reuptake but in a reduced manner (~50% reduction in 5-HTT availability throughout brain regions)¹⁹, as in the case of S carriers in humans^{15,16}. Partial and total 5-*htt* loss-of-function mutations usually are associated with increased serotonin signalling, greater anxiety-like and depression-related behavior as well as heightened response to stress^{4,20}.

Two brain regions that are reached by serotonergic projections and play a key role in regulating emotions and cognitive functions but also neuroplasticity as well as stress, are the hippocampus and frontal cortex²¹. These regions express receptors for hormones and neurotransmitters released following a stressor²¹. The stress response triggers on one hand the activation of the hypothalamic-pituitary-adrenal (HPA) axis with consequent release of corticosteroids (mainly cortisol in humans and corticosterone in rodents), and on the other hand, the sympathetic systems to release catecholamines (e.g. noradrenaline and adrenaline)²². In turn, these hormones and neurotransmitters modulate brain function and behavior by acting on their receptors that are widespread throughout the brain.

To date reviews and meta analyses have questioned the interaction between 5-HTTLPR and stress on depressive symptoms, including the underlying mechanisms^{2, 3, 23}, whereas findings of animal studies have been scattered, and females have often not been included or sex differences have not been investigated^{4, 20}. Therefore, the present study sought to investigate the effect of *5-htt* genotype, early life stress (triggered by maternal separation), and sex, on the expression of candidate genes in the hippocampus and frontal cortex. The expression of HPA axis-related genes (i.e. the arginine vasopressin (*Avp*), proopiomelanocortin (*Pomc*), corticotropin releasing hormone (*Crh*), corticotropin binding protein (*Crhbp*), corticotropin releasing hormone receptor 1 (*Crhr1*)), neuroplasticity-related genes (i.e. brain derived neurotrophic factor (*Bdnf*), neurotrophic receptor tyrosine kinase 2 (*Ntrk2*)), and monoamines metabolism-related genes (i.e. monoamine oxidase a and b (*Maoa*, *Maob*), and catechol-O-methyltransferase (*Comt*)) was assessed in the hippocampus and frontal cortex of male and female rats with normal *5-htt* expression (*5-htt*^{+/+}) and reduced *5-htt* expression (*5-htt*^{+/-}), exposed, or not, to daily maternal separation during the first two postnatal weeks (Figure 1).

Results and Discussion

Results

Gene expression was compared between experimental groups, considering the rearing condition, genotype and sex. *Crhbp* (χ^2 (7) = 13.5, p = 0.01), *Ntrk2* (χ^2 (7) = 15.9, p = 0.026), and *Comt* (χ^2 (7) = 15.2, p = 0.034) were differentially expressed in the hippocampus, whereas *Ntrk2* (χ^2 (7) = 14.3, p = 0.046) and *Comt* (χ^2 (7) = 14.3, p = 0.046) were differentially expressed in the frontal cortex (Figure 2). Nominally significant pair-wise comparisons highlighted differences in the expression of these genes, mainly between *5-htt*^{+/+} rats not exposed to maternal separation and the other experimental groups (Figure S1).

Three-way interaction effects between rearing conditions, genotype and sex were not observed for any gene (p > 0.05) in the hippocampus [*Crh* (F = 0.2; p = 0.668), *Crhr1* (F = 0.9; p = 0.342), *Crhbp* (F = 0.1; p = 0.722), *Bdnf* (F = 1.4; p = 0.241), *Ntrk2* (F = 0.4; p = 0.531), *Maoa* (F = 0.1; p = 0.700), *Maob* (F = 0.4; p = 0.528), and *Comt* (F = 1.7; p = 0.202)] and in the frontal cortex [*Crh* (F = 0.04; p = 0.842), *Crhr1* (F = 0.1; p = 0.771), *Crhbp* (F = 0.5; p = 0.461), *Bdnf* (F = 0.05; p = 0.831), *Ntrk2* (F < 0.001; p = 0.990), *Maoa* (F = 0.1; p = 0.782), *Maob* (F = 0.03; p = 0.863), and *Comt* (F = 0.1; p = 0.701)]. Because of this, males and females were analysed together and the interaction between rearing conditions and genotype studied. Two-way interactions between rearing conditions and genotype were present (Figure 3): in the hippocampus, for *Crhbp* (F = 7.663, η_p^2 = 0.133, p = 0.008; Adj. R^2 = 0.270) and *Ntrk2* (F = 4.797, η_p^2 = 0.088, p = 0.033; Adj. R^2 = 0.195); and in the frontal cortex, for *Crh* (F = 4.1, η_p^2 = 0.077, p = 0.048; Adj. R^2 = 0.141), *Crhbp* (F = 6.743, η_p^2 = 0.121, p = 0.012; Adj. R^2 = 0.115), *Bdnf* (F = 6.884, η_p^2 = 0.123, p = 0.012; Adj. R^2 = 0.137), *Ntrk2* (F = 9.899, η_p^2 = 0.168, p = 0.003; Adj. R^2 = 0.273), *Maob* (F = 7.075, η_p^2 = 0.126, p = 0.011; Adj. R^2 = 0.164), and *Comt* (F = 9.164, η_p^2 = 0.158, p = 0.004; Adj. R^2 = 0.274). In all cases, the expression of these genes was significantly higher in *5-htt*^{+/+} rats not exposed to maternal separation compared to the other experimental groups (Figure 3).

To facilitate comparisons with previous studies as well as future meta-analyses on the effect of maternal separation on gene expression, complementary analyses were performed. In wild-type rats, maternal separation was associated with lower expression levels of the *Crhbp* (U = 18, p = 0.002), *Ntrk2* (U = 23, p = 0.004), *Maoa* (U = 36, p = 0.031), *Maob* (U = 35, p = 0.027), *Comt* (U = 35, p = 0.027) genes in the hippocampus, as well as of the *Crh* (U = 40, p = 0.024), *Crhr1* (U = 36, p = 0.014), *Crhbp* (U = 45, p = 0.045), *Bdnf* (U = 45, p = 0.045), *Ntrk2* (U = 19, p = 0.001), *Comt* (U = 18, p = 0.001) genes in the frontal cortex (Figure S2). On the other hand, rearing condition did not influence gene expression in the *5-htt*^{+/-} rats.

Regarding animals not exposed to maternal separation, *5-htt*^{+/-} compared to *5-htt*^{+/+} control rats displayed lower expression of the *Crhbp* ($U = 5$, $p < 0.001$), *Bdnf* ($U = 14$, $p = 0.004$), *Ntrk2* ($U = 10$, $p = 0.002$), *Maoa* ($U = 9$, $p = 0.001$), *Maob* ($U = 8$, $p = 0.001$), *Comt* ($U = 11$, $p = 0.002$) genes in the hippocampus, as well as of the *Crh* ($U = 28$, $p = 0.035$), *Crhr1* ($U = 18$, $p = 0.006$), *Crhbp* ($U = 21$, $p = 0.01$), *Bdnf* ($U = 13$, $p = 0.002$), *Ntrk2* ($U = 14$, $p = 0.002$), *Maoa* ($U = 21$, $p = 0.01$), *Maob* ($U = 13$, $p = 0.002$), *Comt* ($U = 20$, $p = 0.008$) genes in the frontal cortex. Conversely, hippocampal *Crh* gene expression was higher in *5-htt*^{+/-} compared to *5-htt*^{+/+} control rats ($U = 25$, $p = 0.035$) (Figure S3).

When considering rearing and genotype, correlative relationships were observed following Bonferroni adjustment. Positive strong correlations between expression of the genes ($r > 0.667$) within, but not across, brain regions were found in all groups, except in *5-htt*^{+/+} controls. Few strong negative correlations were present ($r < -0.829$) in the *5-htt*^{+/+} control rats (Figure 4). Sex did not alter these correlative patterns (Figure S4).

Discussion

Both early life stress and constitutive serotonin transporter reduction, independent of sex, were associated with lower expression levels of key genes involved in the stress response system, neuroplasticity and monoamines metabolism. Rats, regardless of sex, exposed to maternal separation and/or carrying the *5-htt*^{+/-} genotype displayed lower mRNA levels of *Crhbp* and *Ntrk2* in the hippocampus, as well as lower mRNA levels of *Crh*, *Crhbp*, *Bdnf*, *Ntrk2*, *Maob*, and *Comt* in the frontal cortex compared to the other groups.

In order to understand the onset of psychiatric disorders, including depression, that are triggered by exposure to stressors and characterized by impaired HPA axis function, we need to advance our knowledge of the neurobiological underpinnings by which the serotonin transporter genotype and early life stress interact to influence brain chemistry. Animal studies enable us to address causality and prospectively investigate the long-term effects of early life stress on health. Indeed, associations with early life stress and maladaptive programming of brain circuits including the regions of interest of the present study have been found, as well as reduced top-down inhibition on the amygdala^{24, 25}. Previous studies have addressed the role of the serotonin transporter and how this genetic factor interacts with environmental stressors in the hippocampus and frontal cortex (reviewed in Houwing et al., 2017²⁰). Gene- and region-specific differential expression was found in the present study.

In line with our previous findings in the prefrontal cortex (PFC)²⁶, *5-htt*^{+/-} rats displayed lower *Bdnf* mRNA levels in the frontal cortex than *5-htt*^{+/+} rats not exposed to maternal separation. In line with other results in the ventromedial PFC²⁷, *5-htt*^{+/-} rats exposed to maternal separation displayed lower *Bdnf* mRNA levels in the frontal cortex compared to non-separated rats, and no additive effect

of serotonin transporter reduction and maternal separation was observed in *5-htt^{+/-}* rats exposed to maternal separation (Figure 3 and S2). BDNF is a neurotrophin playing a role in neuron survival, neurodevelopment and neuroplasticity, whose interaction with serotonin has been demonstrated of relevance to mood disorders by genetic, molecular and pharmacological findings²⁸ (e.g. increased BDNF expression following SSRI treatment²⁹). Lower BDNF expression levels in the frontal cortex, as observed here in the presence of serotonin transporter reduction and maternal separation, may indicate impaired ability of the brain to adapt to the environment³⁰. However, contrary to other studies^{27, 31}, no differences due to genotype by rearing condition were found in *Bdnf* mRNA levels in the hippocampus, though *5-htt^{+/-}* compared to *5-htt^{+/+}* control rats displayed lower hippocampal *Bdnf* mRNA levels. Low hippocampal BDNF levels have been associated with psychiatric disorders and with depression-related behavior in animals exposed to environmental stressors³². In the present study, *5-htt^{+/-}* genotype and/or maternal separation were associated with lower mRNA levels of *Ntrk2* both in the hippocampus and frontal cortex compared to *5-htt^{+/+}* rats not exposed to maternal separation. Given that NTRK2 is the receptor of BDNF, it is plausible to expect similarity in the expression patterns of *Ntrk2* and *Bdnf*, as observed here (Figure 2, 3, S1 and S2); however, no other study investigated this gene in relation to serotonin transporter reduction and maternal separation previously.

Regarding the HPA axis, *Crh* mRNA levels in the frontal cortex followed a similar pattern as *Bdnf* mRNA levels in relation to serotonin transporter genotype and rearing condition (Figure 3 and S2). This is consistent with the lower *Crh* mRNA levels found in the paraventricular nucleus of the hypothalamus of *5-htt^{+/-}* compared to *5-htt^{+/+}* mice³³. On the other hand, we found higher basal hippocampal *Crh* levels in *5-htt^{+/-}* rats compared to *5-htt^{+/+}* rats not exposed to maternal separation, opposite from the effects found in the frontal cortex. Previously no difference in the expression of *Crh* levels was found in the bed nucleus of the stria terminalis and central amygdala between *5-htt^{+/-}* and *5-htt^{+/+}* rats, exposed or not to early life stress³⁴, thus suggesting that the *5-htt^{+/-}* genotype affects *Crh* expression levels in a brain region-specific manner. This has previously also been found regarding *Bdnf* expression, where *Bdnf* expression was increased due to stress in the dorsal hippocampus and dorsomedial prefrontal cortex of *5-htt^{+/-}* rats, but decreased due to stress in the ventral hippocampus of *5-htt^{+/-}* rats²⁷. Moreover *Bdnf* levels were lower in the ventromedial prefrontal cortex of *5-htt^{+/-}* compared to *5-htt^{+/+}* rats²⁷. The anatomy-dependent expression profile found for *Bdnf*, but also the diverse *Crh* expression across brain areas found in the present study, might pinpoint alterations in mechanisms underlying behavioural outcomes due to reduced *5-htt* expression or early life stress that are brain region-specific. Differences in hormone levels and expression of genes related to the HPA axis have also been identified in previous studies on serotonin transporter reduction and maternal separation, as reviewed by Houwing²⁰. Discrepancies in findings

may be explained by compensatory mechanisms taking place downstream in the HPA axis³⁵. Corticolimbic structures also express receptors for CRH, with the hippocampus and frontal cortex exerting inhibitory effects on the HPA axis³⁵; thus lower *Crh* mRNA levels in the frontal cortex could lead to hyperactivation of the HPA axis. Maternal separation for 180 minutes has indeed been associated with elevated corticosterone levels³⁶, likely resulting from the disruption of the HPA axis negative feedback. By binding to CRHR1, which is expressed in brain regions associated with affective and stress circuitries³⁷, CRH is known to be involved in phenotypical changes caused by early life stress. Contrary to other studies, we found lower *Crhr1* mRNA levels in the frontal cortex of non-stressed *5-htt*^{+/+} rats compared with maternally separated *5-htt*^{+/+} and non-stressed *5-htt*^{+/-} rats, following the *Crh* expression pattern (Figure S2). Similar to the findings of O'Malley and colleagues³⁸, we found no differences in *Crhr1* expression in the hippocampus. Interestingly, the study of O'Malley investigated protein levels confirming the present mRNA data. Activation of the *Crh* receptors is regulated by the secretory glycoprotein *Crh*-binding protein (*Crhbp*)^{39,40}, and interestingly we show that *Crhbp* mRNA levels resemble the *Crh* expression levels; they were lower in the presence of maternal separation and *5-htt*^{+/-} genotype (Figure 2, 3, S1 and S2).

The stress system comprises not only the HPA axis but also the sympathetic branch of the autonomic nervous system whose role in the response to stressors takes place through the release of catecholamines, such as norepinephrine and epinephrine. Catecholamines, including dopamine, are metabolized by COMT, which has received attention with regard to its role in stress vulnerability⁴¹. The common variant coding a valine (Val, G allele) in the protein sequence of *COMT* leads to higher COMT activity than the methionine (Met, A allele)⁴². This specific *COMT* Val158Met polymorphism has been linked to emotional and behavioral disorders, and maternal stress has been shown to increase the risk of childhood behavioral problems in Met/Met carriers of the Val158Met polymorphism⁴³. The present study showed that *5-htt*^{+/+} rats exposed to maternal separation showed lower *Comt* expression in the hippocampus as well as frontal cortex (Figure S2), suggesting that *Comt* expression might be involved in phenotypical changes induced by early life stress. Moreover, compared to *5-htt*^{+/+} rats, *5-htt*^{+/-} rats displayed lower *Comt* expression in both the frontal cortex and hippocampus (Figure 2, 3 and S1). Regarding the metabolism of monoamines, the gene expression of *Maob* in the frontal cortex resembled the gene expression of *Comt*; a lower gene expression of *Maob* in male and female rats, both maternally separated as well as with the *5-htt*^{+/-} genotype (Figure 3). Interestingly the metabolism of serotonin is also served by monoamine oxidases, even though *Maob* has a lower affinity to serotonin compared to *Maoa*⁴⁴. Maternal separation in *5-htt*^{+/+} rats was associated with lower *Maoa* and *Maob* in the hippocampus (Figure S2), whereas the *5-htt*^{+/-} genotype displayed lower gene expression of *Maoa* and *Maob* in both the hippocampus and frontal cortex. Disruption of MAOA and MAOB has been implicated in behavioral

1
2
3 abnormalities⁴⁵. Although basal extracellular levels of 5-HT in the brain are not different in *5-htt*^{+/-}
4 rats compared to *5-htt*^{+/+} rats, maternal separation might have challenged the monoaminergic
5 neurotransmission homeostasis underpinning neuropathological changes, especially due to the
6 neurotrophic actions of 5-HT. In fact, knowing that the serotonin transporter is responsible for
7 terminating the serotonin signal at the synaptic level through a reuptake mechanism⁴⁶, any
8 difference in its expression and function can indeed influence serotonin-related physiology, with
9 repercussions for neuroplasticity, emotional and cognitive processing²¹.

15
16 Correlative relationships dependent on genotype and rearing condition were observed. The
17 presence of rather strong positive correlations between the expression of stress-related genes within
18 brain regions in rats carrying the *5-htt*^{+/-} genotype and/or exposed to maternal separation suggests a
19 pattern of molecular changes orchestrated by the genetic make-up and environment because this
20 was not observed in *5-htt*^{+/+} rats unexposed to maternal separation. Interestingly, while less positive
21 correlations survived correction for multiple testing in *5-htt*^{+/-} rats exposed to maternal separation,
22 the combination of these two factors led to the emergence of positive gene expression correlations
23 also between brain regions. This could be interpreted as an additional GxE interaction at the brain-
24 system level; indeed, both the frontal cortex and the hippocampus exert inhibitory control on the
25 HPA axis.

32
33 Translational models of constitutive serotonin transporter reduction and experimentally
34 controlled stressors have so far produced inconsistent results, often depending on the behavior, or
35 anatomical and molecular substrate analyzed. Moreover, limited designs have undermined several
36 studies, as reviewed by Houwing and colleagues²⁰. The findings of the present study should also be
37 considered in light of its methodological strengths and limitations. While differences in sample size,
38 study design, definition of environmental and phenotypic variables, and statistics have hampered
39 interpretation and generalizability of the results of human studies^{4, 23}, animal models as the current
40 one present several advantages. The genetic make-up can be experimentally manipulated and
41 controlled, likewise the rearing environment; however, this limits the influence of potential
42 confounding factors that are present throughout life and influence phenotypic outcomes^{4, 47, 48}.

50
51 In the present study, we employed the *5-htt*^{+/-} rats as a translational model for the human s-
52 allele carriers to study the underlying molecular mechanisms involved in early life stress by *5-htt*
53 interactions. Though expression of *5-htt* was not measured in the present study, *5-htt*^{+/-} rats have
54 been shown to display a ~50% reduction in 5-HTT availability throughout brain regions¹⁹, in line with
55 *in vitro* studies of the *5-HTTLPR*^{15, 16}. In humans, the *5-HTTLPR* polymorphism has been poorly
56 investigated in terms of serotonergic function. In healthy adults, no association has been observed
57 between *5-HTTLPR* and its availability in the brain measured by positron emission computed
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tomography⁴⁹ or the concentration of the serotonin metabolite 5-HIAA in the cerebrospinal fluid⁵⁰. It is likely that the influence of constitutive serotonin transporter reduction in the brain is mostly exerted during early development, on neurogenesis and differentiation processes, and then partly buffered or modified by other factors throughout development and aging^{10, 51, 52}, potentially explaining the non-additive effect observed at the molecular level in the present study.

The interaction between the serotonin transporter genotype and stress has been linked to depression¹⁻³, and sensitivity to early-life stressful events has been shown in *5-htt*^{+/-} male⁵³ and female⁵⁴ rats. In the present study, emotional and physical neglect early in life was simulated by making use of the maternal separation model during the first postnatal weeks⁵⁵. Prolonged lack of maternal care can influence brain development negatively, as proven by its association with differential molecular, neuroendocrine and behavioral outcomes⁵⁶⁻⁵⁸ of translational relevance to psychiatric outcomes in humans exposed to early-life stressors^{59, 60}. Although duration, frequency, timing and regularity of maternal care disruption are factors that preclude comparisons between preclinical studies^{58, 61-63}, maternal separation for 180 minutes during the first two postnatal weeks, as performed in the present study, is considered a valid stressor for the pups⁶⁴. Its occurrence during the so-called stress hypo-responsive period can disrupt the development of the HPA axis^{65, 66}. However, as observed here, the effect of maternal separation may be buffered at the molecular level in individuals already genetically predisposed, such as *5-htt*^{+/-} rats, to impaired HPA axis function. The present results, in fact, suggest a link between 5-HTT alterations and maternal separation as patterns of expression of stress-related genes in the hippocampus and frontal cortex of rats with constitutive serotonin transporter reduction resembled the ones displayed by rats exposed to maternal separation. This is in line with previous studies demonstrating an impact of maternal separation on serotonergic function⁶⁷⁻⁷². In the dorsal raphe nucleus, a region not investigated in the present study, lower expression of *5-htt* mRNA has been found in rats exposed to maternal separation (MS180⁶⁷ and MS 15⁶⁸) and lower 5-HTT binding potential has been found in peer-reared monkeys exposed to maternal deprivation⁷²; these animals also showed depression and anxiety-like behavior^{67, 72}. Moreover, postnatal treatment with clomipramine, leading to depression-like behavior, has been associated with low *5-htt* mRNA levels in the DRN⁷³. Further studies should investigate the combined effect of serotonin transporter reduction and maternal separation on behavioral and neural outcomes.

A stressful environmental factor was considered in the present study. Interestingly, various psychobiological theories have been applied to the 5-HTTLPR x environment field of research (e.g. the diathesis-stress⁷⁴, vantage-sensitivity⁷⁵, match/mismatch⁷⁶, differential susceptibility⁷⁷ and biological sensitivity to context⁷⁸). In line with the theories posited by Boyce and Belsky^{77, 78}, the S

allele of the serotonin transporter gene has been more recently suggested as a plasticity allele associated with increased sensitivity to protective and stressful environmental factors in relation to symptoms of depression^{79, 80}. Indeed, depending on 5-HTTLPR genotype, the same individual may thrive and suffer in the presence of supportive and aversive environmental influences, respectively⁸¹, as recently demonstrated in monkeys carrying the short allele⁸². A beneficial environment in rodents can be modelled by environmental enrichment, and its interactive effect with early life stress has recently been demonstrated on the pups' behavior and brain chemistry⁸³, thus calling for studies considering also positive environmental factors.

Additionally, in contrast to previous studies²⁰, both sexes were here investigated, although no difference was observed between males and females. The serotonergic system as well as the stress system have been shown to interact with sex⁸⁴. For instance, lower serotonin synthesis rates have been found in the brain of females compared to males⁸⁵, and higher levels of the serotonin metabolite 5-HIAA have been observed in women carrying the S allele⁸⁶. Moreover, studies of humans reported higher risk for depression, in the context of exposure to stressors, to be restricted to females carrying the S allele of the 5-HTTLPR⁸⁷⁻⁹¹ or that the L allele is the risk factors in males^{88, 92-95}. It could be plausible to hypothesize that behavioral outcomes measured following puberty may have differed between the sexes, as often observed in humans⁹⁶. Nevertheless, the present study found no sex difference at the molecular level when the stressor was applied in the first two postnatal weeks. Missing data on immobility-induced swim stress behavior could have informed us about differences in coping style. While this limits our interpretation, increased immobility behavior has been associated with a full 5-htt loss-of-function mutation, independent of sex⁹⁷. However, in mice no differences were found at basal levels or after a shock-stressor in forced swim immobility time between 5-htt^{+/-} and 5-htt^{+/+} genotypes⁹⁸, although a tendency to reduced mobility time after prenatal stress exposure was found in female 5-htt^{+/-} compared to 5-htt^{+/+} mice⁹⁹. Even though we cannot draw any conclusion from the forced swim test performed in the present study, it should be noted that the swim stress could have potentially affected the expression of the genes in the prefrontal cortex and hippocampus. As part of the test, rats were placed in water tanks for a total of twenty minutes (i.e. 15 during the induction phase on day 1 and 5 during the test day 24 h later). While effects of housing, maternal separation and other stressors on gene expression in the brain have been studied and demonstrated; the impact of such a time-limited experience remains to be investigated.

Conclusions

The present findings indicate differential expression of genes involved in the stress response system, neuroplasticity and catecholamine metabolism, in the hippocampus and frontal cortex, due to

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2
3 reduced serotonin transporter expression and/or following maternal separation, but independent of
4 sex. No additive effect of serotonin transporter reduction and maternal separation on gene
5 expression was found; such floor effects might explain inconsistent findings in humans and rodents.
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Methods

Animals

The study design is presented in Figure 1. Wildtype and heterozygous serotonin transporter knockout rats (*Slc6a4*^{1Hubr}; *5-htt*^{+/+} and *5-htt*^{+/-}) were derived from crossing female wildtype Wistar (WU, Harlan) with *5-htt*^{+/-} and *5-htt*^{-/-} male rats (GenOway, Lyon, France). The pregnant dams were housed in standard polysulfone cages (40 × 20 × 18 cm) containing wood chip bedding material and two paper sheets (40 × 60 cm; Cellstoft Papyrus) as enrichment. Rats had *ad libitum* access to water and food (R36; Lantmännen, Kimstad, Sweden) in a temperature (21 ± 1 °C) and humidity-controlled room (45% – 60% relative humidity), with a 12 h light/dark cycle (lights on at 7:00 a.m.). The dams were inspected daily for delivery of pups at 5:00 p.m., and day of birth was designated as postnatal day (PND) 1. All experimental procedures were approved by the Uppsala Animal Ethical Committee and followed the guidelines of the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC).

Maternal separation

Litters were randomly allocated to one of two rearing conditions (from PND 2 to 15): maternal separation for 180 min (MS180) or no maternal separation (MS0). MS180 was started daily between 8:30 and 9:00 a.m., and was performed as follows: the pups were removed from the home cage and placed as a whole litter into a smaller cage (24 × 15 × 14 cm) with only sawdust bedding after which they were transferred to a temperature incubator in adjacent room. The temperature was set to maintain a temperature of 29 ± 1 °C. At the end of the separation period, litters were returned to their home cage by placing them in the nest and covering them with home cage bedding material. Home cages were refreshed at PND 7 and 14. At PND 21, ear punches were taken of the pups for identification and genotyping. At PND28, the pups were weaned and housed in groups of three to four littermates of the same sex and rearing, under the same conditions as mentioned above.

Genomic DNA isolation

Ear punches were lysed overnight in 400 µL of lysis buffer, containing 100 mM Tris (pH 8.5), 200 mM of NaCl, 0.2% of sodium dodecyl sulfate (SDS), 5 mM of ethylene diamine tetraacetic acid (EDTA), and 100 µg/ml of freshly added Proteinase K at 55°C. The next day, Proteinase K activity was ended by 10 min incubation at 80°C. Samples were cooled and shortly centrifuged to collect condensate. DNA was precipitated by adding 400 µL isopropanol, mixing by inversion, followed by centrifuging for 10 min at 14.000g. The supernatant was removed by gently inverting the tube and

the pellets were washed with 300 μ L 70% ethanol, centrifuged for 5 min at 14.000 g. The supernatant was discarded with a pipette, pellet air-dried and the DNA solved in 100 μ L TE-buffer by incubating 10 min at 70°C and vortexing.

Genotyping

The following primers and probes were used: Forward primer: 5'-GCACGAACTCCTGGAACACT, Reverse primer: 5'-AGCGTCCAGGTGATGTTGTC, Serotonin wildtype probe: 6FAM-AGTTGGTGCAGTTGC-MGBNFQ; Serotonin transporter knockout probe: VIC-AGTAGTTGGTTCAGTTGC-MGBNFQ (solved in 20x primer solution, Life technologies, Sweden). Genotyping was performed using Applied Biosystem step one plus (Life technologies, Sweden). The total reaction was 25 μ L containing 12.5 μ L Taqman Universal Mastermix II, no UNG (cat# 4440047, Life Technologies, Sweden); 1.25 μ L 20x primer solution; 10.25 μ L sterile H₂O and 1 μ L DNA sample. The thermal cycling for genotyping was as follows: 95°C 10 min +40 \times [92°C 15 s + 60°C 1min]. Genotypes, *5-htt*^{+/+} and *5-htt*^{+/-}, were manually inspected comparing them with positive controls.

Tissue collection

A forced swim test was performed at day 84 (15 min) + day 85 (5 min) to address immobility. Two weeks after the forced swim test, at PND98, rats were sacrificed by decapitation. Rats were taken from their home cage into a separate room and decapitated within 10 s. Immediately after decapitation, the frontal cortex (brains were put in a brain grid and the frontal part of the brain was cut at approximately Bregma 3.20 mm (Paxinos and Watson Atlas), subsequently the PRL/CG1/M2 part was roughly dissected) and hippocampus were isolated and immediately frozen in liquid nitrogen and stored at -80 °C until further use. Note: half of the videos for the forced swim test cannot be retrieved due to saving errors. Therefore, data of the forced swim test are not shown.

Total RNA Isolation

Total RNA was isolated from brain tissue using an RNeasy lipid tissue mini kit (#cat 74804; Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was evaluated using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA).

cDNA synthesis

Total RNA (250 ng) from each sample was used to generate complementary DNA (cDNA) using Superscript VILO Mastermix (#cat 11755-250; Invitrogen, Carlsbad, CA) according to the

manufacturer's protocol. Briefly, 4 μ L Superscript VILO Mastermix was added to 250 ng RNA and filled to a volume of 20 μ L with DEPC-treated water. The thermal cycling for cDNA synthesis was as follows: 25°C 10 min + 42°C 60 min + 85°C 5min. The cDNA samples were stored at -20°C until quantitative PCR analysis.

Quantitative PCR

The relative expression of *Avp*, *Pomc*, *Crh*, *Crhbp*, *Crhr1*, *Bdnf*, *Ntrk2*, *Maoa*, *Maob*, and *Comt* was investigated. Quantitative PCR (qPCR) reactions were carried out in a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were performed in MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml and appropriate MicroAmp® Optical Adhesive Film (Applied Biosystems, Foster City, CA). The reaction mixture consisted of 10 μ L TaqMan gene expression Mastermix (Applied Biosystems, Foster City, CA, USA), 1.0 μ L cDNA, and 1 μ L 20x primers (Life Technologies, Sweden). The final reaction volume was 20 μ L. The thermal cycling for quantitative PCR was as follows: stage 1: 2 min at 50 °C + 10 min 95 °C; stage 2: 55 cycles of denaturation at 95 °C for 15 s and combined primer annealing/extension at 60 °C for 1 min. The primers are shown in Table S1.

Relative fluorescence values were converted into quantification cycle (Cq) values, the cycle at which the gene reached a detection threshold, controlling for batch effects. The LinReg software¹⁰⁰ was used to compute the batch-averaged baseline and qPCR efficiencies (Table S2). Cq values were adjusted across the plates using correction factors derived from threshold and PCR efficiency values. Each sample was run in triplicates. Samples with normalized Cq values that had a standard deviation of more than 0.5 were excluded. Following computation of the average of the triplicates for each sample, the mean of the average of both reference genes (*Gapdh* and *Ywhaz*) was used to estimate the reference Ct values. Relative gene transcripts levels were determined using the Δ CT method (Real-Time PCR Applications Guide, Bio-Rad®). All the laboratory and pre-processing analyses were performed in a blind manner. Valid gene expression data were retrieved for the frontal cortex of n = 4 female MS0-5-*htt*^{+/+}, n = 6 female MS0-5-*htt*^{+/-}, n = 8 female MS-5-*htt*^{+/+}, and n = 8 female MS-5-*htt*^{+/-}, and n = 6 male MS0-5-*htt*^{+/+}, n = 6 male MS0-5-*htt*^{+/-}, n = 9 male MS-5-*htt*^{+/+}, and n = 6 male MS-5-*htt*^{+/-} rats and for the hippocampus of n = 4 female MS0-5-*htt*^{+/+}, n = 5 female MS0-5-*htt*^{+/-}, n = 6 female MS-5-*htt*^{+/+}, and n = 8 female MS-5-*htt*^{+/-}, and n = 6 male MS0-5-*htt*^{+/+}, n = 6 male MS0-5-*htt*^{+/-}, n = 9 male MS-5-*htt*^{+/+}, and n = 10 male MS-5-*htt*^{+/-} rats.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS) software version 22 (SPSS Inc., IBM SPSS Statistics, Chicago) was used for statistics analyses. Normality of the gene expression data was tested

using the Shapiro Wilk test. Non-parametric tests were performed to account for deviation from normal distribution. Differences in gene expression between groups were assessed using the Kruskal-Wallis and Mann-Whitney U tests. Interactions effects between rearing conditions, genotype and sex on gene expression were tested using the general linear model (GLM) with type III sum of squares. Correlations between the expression of the genes were analyzed using the Spearman correlation test. Correction for multiple comparisons was performed using Bonferroni correction.

Supporting Information

Table S1. Specifics of TaqMan® Gene Expression Assays; Table S2. Mean qPCR efficiency and Cq range for the genes of interest; Figure S1. Pair-wise, nominally significant differences in relative gene expression were observed between male and female 5-htt^{+/+} rats not exposed to maternal separation and the other experimental groups.; Figure S2. Maternal separation-driven relative gene expression differences in 5-htt^{+/+} rats; Figure S3. Serotonin transporter genotype-driven relative gene expression differences in rats not exposed to maternal separation; Figure S4. Correlations heat maps of relative gene expression by experimental group and sex.

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The authors declare no conflict of interest.

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Figure 1

Experimental design. Rats were exposed to daily maternal separation (MS) for 180 minutes or no maternal separation (MS0) during the first two postnatal weeks. The Forced Swim Test (FST) was assessed during adulthood. Gene expression analyses were performed on hippocampus and frontal cortex tissue dissected on postnatal week 14.

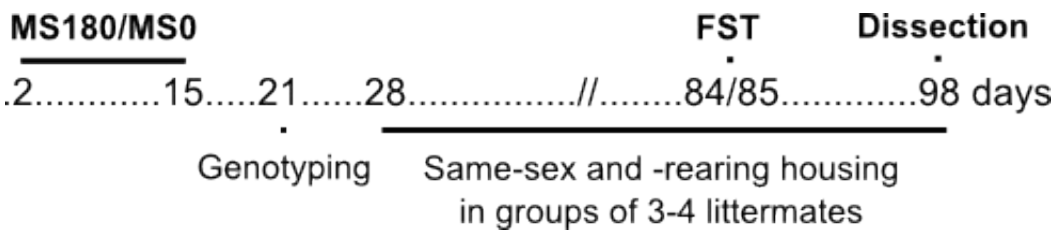
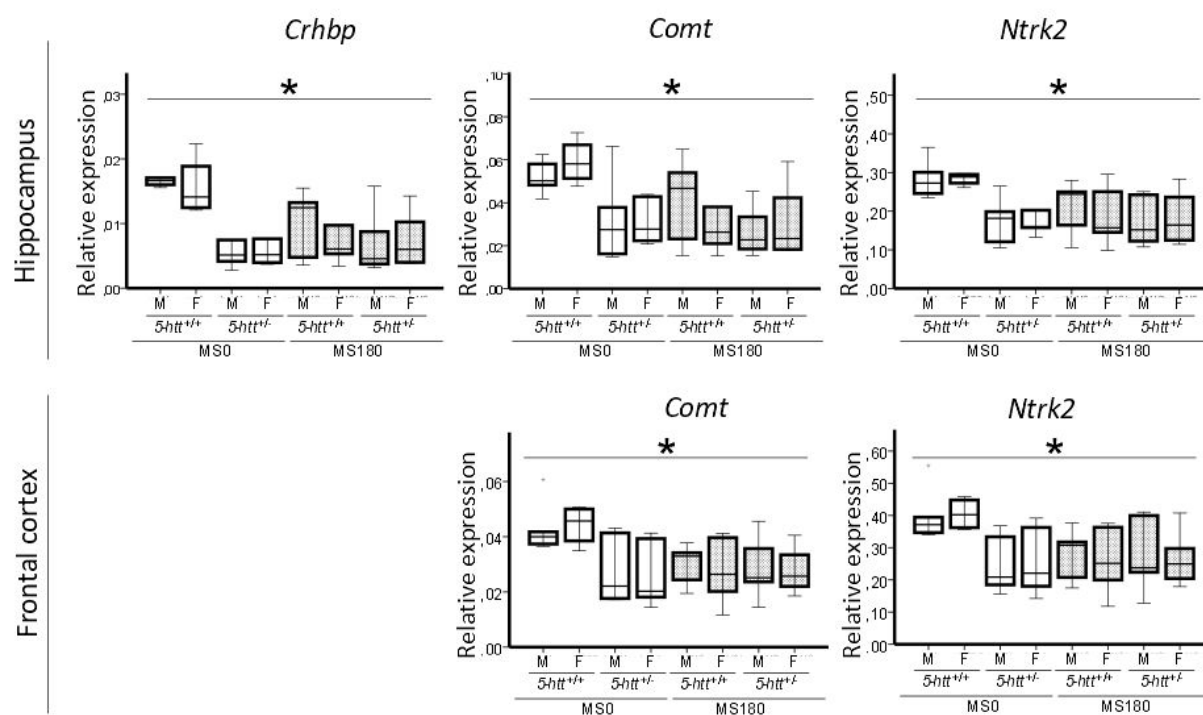


Figure 2

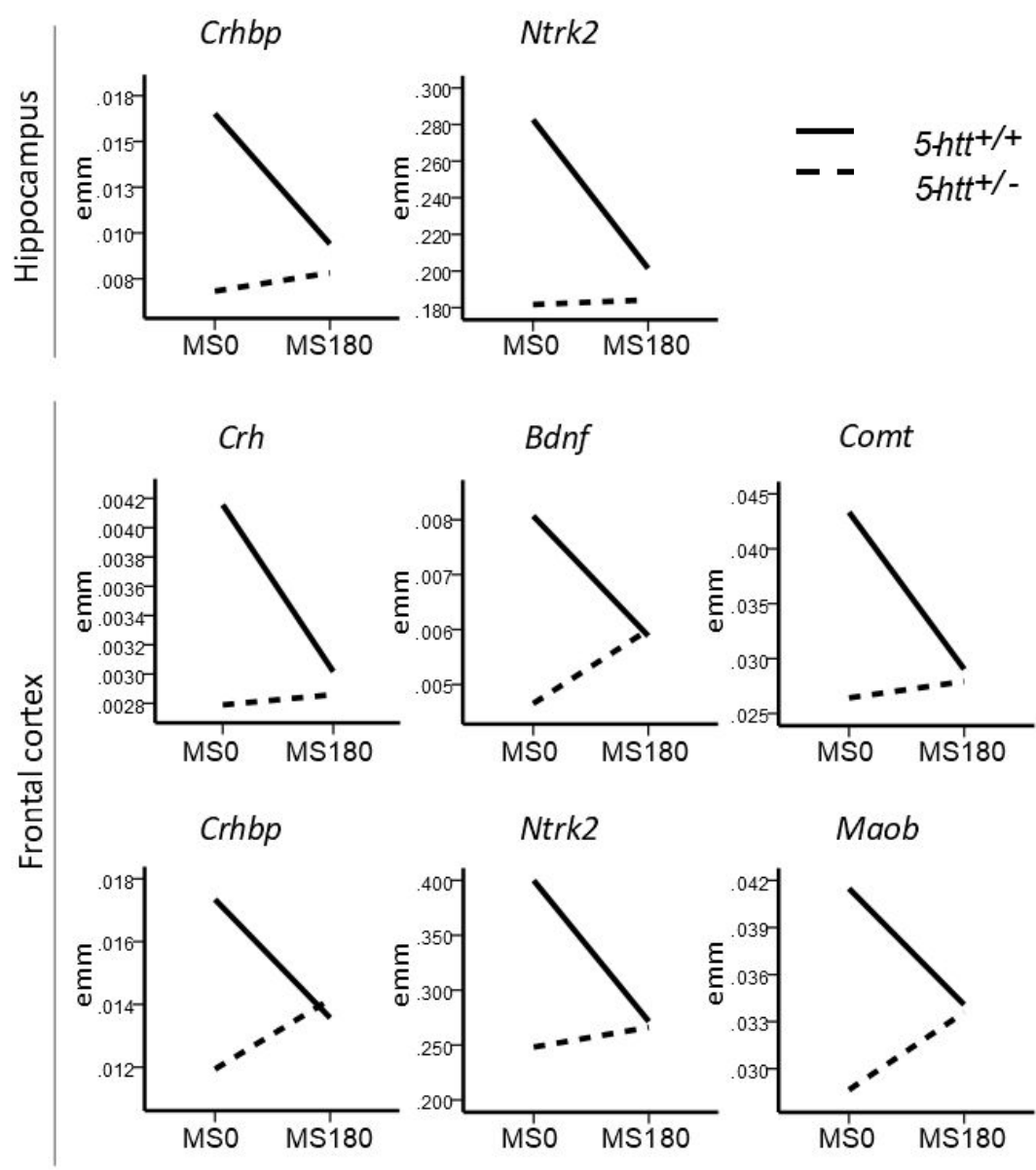
Between-group differences in relative gene expression, considering genotype, rearing condition and sex. Pair-wise comparisons are illustrated in Figure S1.



F: females; M: males; MS0: no maternal separation; MS180: maternal separation for 180 minutes; *: Kruskal-Wallis test $p < 0.05$

Figure 3

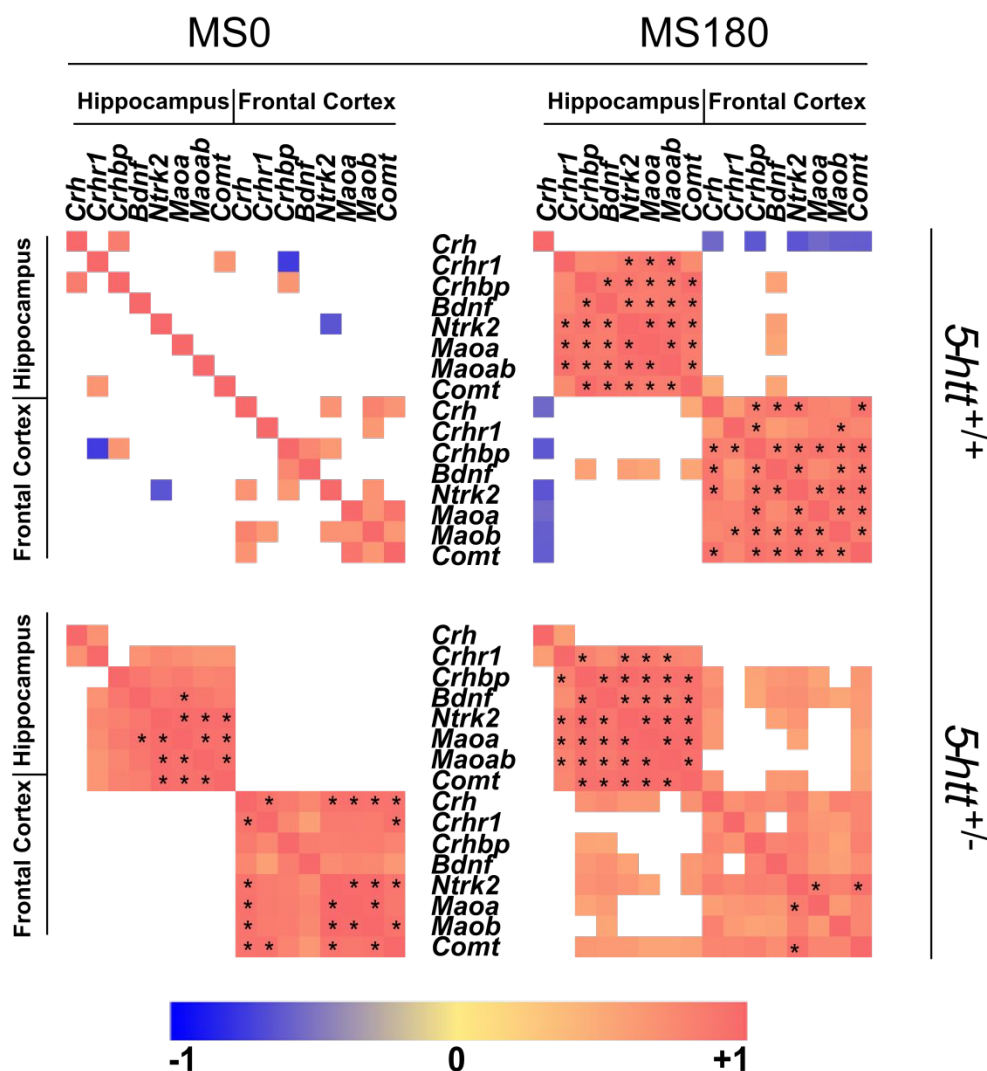
Genotype by rearing condition interaction plots of the estimated marginal means of relative gene expression profiles in the hippocampus and frontal cortex.



emm: estimated marginal means; MS0: no maternal separation; MS180: maternal separation for 180 minutes

Figure 4

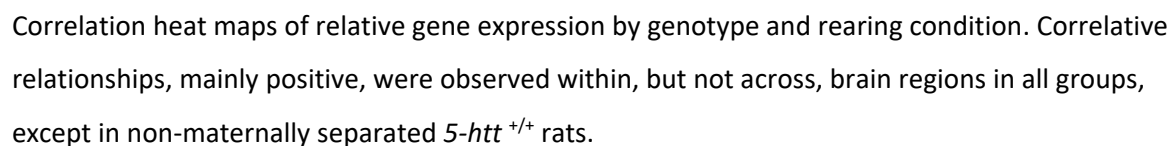
Correlations heat maps of relative gene expression by genotype and rearing condition.



The color bar indicates the Spearman's ρ ; *: significant correlations after correction for multiple testing, Spearman test $p < 0.0001$; MS0: no maternal separation; MS180: maternal separation for 180 minutes

Constitutive serotonin transporter reduction resembles maternal separation with regard to stress-related gene expression

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The color bar indicates the Spearman's ρ ; *: significant correlations after correction for multiple testing, Spearman test $p < 0.0001$; MS0: no maternal separation; MS180: maternal separation for 180 minutes